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Journal

The Journal of experimental medicine, 177(3)

ISSN

0022-1007

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Publication Date

1993-03-01

DOI

10.1084/jem.177.3.729

Peer reviewed

Ordered Rearrangement of Variable Region Genes of the T Cell Receptor γ Locus Correlates with Transcription of the Unrearranged Genes

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Summary

T cell receptor Vγ genes rearrange to the Jγ1 gene segment in a highly ordered fashion during development. We demonstrate a striking correlation between the pattern of expression of unrearranged Vγ genes and the timing of their rearrangement. Thus, the increases in Vγ2 rearrangements, and decreases in Vγ3 and Vγ4 rearrangements observed during development are paralleled by increasing or decreasing levels of the corresponding unrearranged V gene transcript. We also provide evidence that both the Vγ3 and Vγ4 genes are accessible in mature Vγ3$^+$ cells, but that the Vγ4 gene may be inaccessible in the progenitors of Vγ3 cells. The results suggest that regulated local accessibility of the chromatin surrounding Vγ genes is responsible for ordered Vγ gene rearrangement during development.

γ/δ T cells can be categorized into subtypes differing in location, receptor structure, and extent of receptor diversity (1–3). The differences between subtypes suggest they are specialized for different functions. Intraepithelial lymphocytes in the epidermis (s-IELs)[1] and the female reproductive tract (r-IELs) have homogeneous receptors. Virtually all s-IELs have a receptor composed of Vγ3-Jγ1Cγ1/Vδ1-Dδ2-Jδ2Cδ chains, with the identical coding sequence at their V-(D)-J junctions (4, 5). These cells respond in vitro to an antigen expressed on stressed keratinocytes (6), and may function to remove old or damaged skin cells. r-IELs express the same rearranged δ gene sequence as s-IEL, which is paired with an invariant Vγ4-Jγ1 chain (7, 8). Their function is unknown, but given the similarities to Vγ3$^+$ cells, may involve recognition of epithelial cells resident in the female reproductive tissues.

γ/δ cells in the secondary lymphoid organs are the most diverse, utilizing several Vγ genes (Vγ2, Vγ1.1, and Vγ1.2) and Vδ genes, in which junctional diversity is extensive (9–11). This population includes γ/δ cells specific for classical and nonclassical MHC antigens (12–14), and it has been suggested that these cells may have the capacity to respond to conventional antigens (1, 14).

During thymic ontogeny, the different γ/δ subtypes arise in ordered waves. This is evident from analyses at the level of TCR gene rearrangements and at the level of cell surface TCR expression. Vγ3 and Vγ4 genes, which are most proximal to the Jγ1 gene segment, are commonly rearranged in the fetus, but rarely so in the adult. In contrast, rearrangements of the more 5′ Vγ2 are relatively rare in the early fetal thymus and predominate in the adult (1, 15–17). At the δ locus, Vδ1 rearrangements predominate in the fetus (18), whereas other Vδ regions, especially Vδ5, predominate in the adult (9, 19).

In parallel to the corresponding gene rearrangements, the first wave of thymocytes, bearing a Vγ3/Vδ1 receptor, appears at approximately fetal day 13 (E13), peaks at E15, decreases by E18, and is nearly undetectable in the adult (20–22). The Vγ4/Vδ1 cells are thought to arise in a slightly later wave, which fades by the adult stage. However, the lack of Vγ4-specific antibodies prevents an exact comparison of the Vγ3 and Vγ4 waves (23). In contrast, thymocytes bearing polymorphic Vγ2 receptors first arise around E16, and increase to become a major γ/δ subtype in the adult thymus (21, 22).

The basis of the ordered appearance of γ/δ subtypes is poorly understood. Evidence indicates that the stage of development of both the thymic stroma and the T-progenitor are important, since differentiation of Vγ3$^+$ cells occurs efficiently only if both the thymic stromal tissue and the T cell progenitors are of fetal origin (24, and J. P. Allison, personal communication). It is possible that stage-specific thymic ligands select for distinct γ/δ subtypes that arise more or less randomly. Alternatively, stage-specific thymic signals may stimulate production of distinct γ/δ cell subtypes during ontogeny. The latter model predicts that rearrangement of distinct γ (and δ) genes is not random, but is programmed by the thymic environment and/or the origin of the progenitor cell. We have argued elsewhere in favor of a targeted gene

[1] *Abbreviations used in this paper:* r-IEL, intraepithelial lymphocytes in the female reproductive tract; s-IEL, intraepithelial lymphocytes in the epidermis.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/03/0729/11 $2.00
Volume 177 March 1993 729–739

rearrangement model (25), based in part on the observation that γ gene rearrangements on the second, unused chromosome of cloned γ/δ^+ cell lines tend to be of the same type as found on the productively rearranged chromosome.

The ordered pattern of Vγ gene rearrangement in developing T cells is similar in some respects to the rearrangement of Ig V_H genes in developing B cells. V_H gene rearrangement also occurs in an ordered manner, with the most 3′ segments rearranging earlier and 5′ segments later (26–30). The generality of this phenomenon suggests that similar mechanisms regulate rearrangement in the different gene families.

Several instances have been reported where rearrangement is correlated with prior transcription of unrearranged genes. For example, transcripts of unrearranged V_HJ558 genes appear in developing fetal liver pre-B cells immediately before rearrangement of the corresponding genes (31, 32). In the case of a recombination substrate that has been transfected into a pre-B cell line, selection for transcription of a linked gene results in high rates of subsequent rearrangement (33). Induction of κ gene rearrangement in pre-B cell lines correlates with expression of unrearranged Cκ and Vκ genes (34, 35). Rearrangement of a transgenic recombination substrate occurs efficiently only in the presence of an active transcriptional enhancer (36). In recent studies of the H chain class switch, transcription of downstream C_H genes, induced by cytokines, immediately precedes switch recombination to that constant region gene (37, 38). And rearrangement of TCR Vα genes by a cell line in culture correlated with transcription of the unrearranged genes (39). It has been proposed that the accessibility of a gene to the recombinase machinery may be controlled, at least in part, by transcription (33). Alternatively, accessibility of an unrearranged gene may be activated at another level, resulting in increases in both transcription and rearrangement of the gene (40–42).

The accessibility model has considerable experimental support in both V-(D)-J rearrangement and Ig H chain class switching. However, in no case has it been shown that ordered V gene rearrangement patterns in development can be correlated with differential accessibility of the corresponding V genes. We have examined the ordered rearrangement of a subset of the Vγ genes, Vγ3, Vγ4, and Vγ2, which rearrange in a highly ordered fashion to the single Jγ1 gene segment of the Cγ1 gene. Here we demonstrate a striking developmental relationship between the appearance and disappearance of sterile Vγ transcripts and the timing of the corresponding Vγ gene rearrangement in thymocytes. These findings suggest that ordered Vγ gene rearrangement is accomplished by the selective induction of V-gene accessibility, which is correlated by transcription of the unrearranged genes.

Materials and Methods

Mice. BALB/c-ByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Fetal mice were bred at the University of California, Berkeley.

Antibodies. Monoclonal anti-CD8 (AD4(15) [43]) and anti-CD4 (GK1.5 [44]) antibodies were used in the form of culture supernatants. PE-conjugated anti-CD3ϵ (500-A2) was purchased from Pharmingen, Inc. (San Diego, CA).

Cell Lines. The 7.17.A2 cell line ([45] kindly provided by R. E. Tigelaar, Yale University, New Haven, CT) was grown in RPMI-1640 supplemented with 5% FCS, 50 μM 2-ME, 0.2 M Hepes, antibiotics, and 20 U/ml hu-rIL-2 (Cetus Corp., Emeryville, CA).

Thymocyte Isolation. Gestational age of fetuses was determined as the number of days after the morning of appearance of a vaginal plug (day 0). Total thymocytes were used from embryonic days 14 and 15 (E14, E15) fetuses. $CD4^-CD8^-$ thymocytes were isolated from E18 and adult (3–4-wk-old) mice, by treatment of the cells with anti-CD4 and anti-CD8 antibodies plus complement (a mixture of rabbit [Cedarlane Laboratories, Ontario, Canada] and guinea pig [Gibco Laboratories, Grand Island, NY] sera [46]). Fetal thymocytes were pooled from several litters and adult thymocytes from at least 10 animals. For E15, E18, and adult mice, debris from the disrupted thymus capsules was allowed to settle out during thymocyte isolation. This step was omitted when isolating E14 thymocytes to increase cell yield.

$CD3^-$ cells were prepared as follows: total thymocytes from E15 or $CD4^-CD8^-$ thymocytes from adult mice were stained with PE-conjugated antibody against the TCR-associated CD3-ϵ chain. The $CD3^-$ cells were purified by electronic cell sorting using a FACS IV® (Becton Dickinson & Co., Mountain View, CA). Dead cells were gated out on the basis of forward scatter and propidium iodide uptake.

Nucleic Acid Preparation. Genomic DNA was isolated from thymocytes as described (47). Total RNA was isolated using the guanidinium/CsCl method (48). DNA fragments corresponding to rearranged and unrearranged Vγ genes were subcloned into plasmids (Gemini; Promega Biotech, Madison, WI), to allow the production of DNA and RNA templates of known quantities used to standardize the quantitative PCR experiments. Synthetic RNAs used as standards for absolute quantitation were transcribed in vitro using T7 or SP6 RNA polymerase (Promega Biotech). All RNA samples were treated by digestion with RQ-1 RNase-free DNase (Promega Biotech) to remove any contaminating genomic or plasmid DNA. RNA and DNA samples were quantitated spectrophotometrically and their condition observed on ethidium bromide–stained agarose gels.

Southern Blot Analysis. Southern blot analysis was as described (15) with the following exceptions: prehybridization wash was omitted and after hybridization, filters were washed twice with 2× SSC, 0.05% SDS at room temperature and twice with 0.1× SSC, 0.05% SDS at 60°C.

PCR Primers. Synthetic oligonucleotides used as primers are as follows: CTGGGAATTCAACCTGGCAGATG (L2), GCTAAGAAGGATGTGGGTTG (V2-3′a), CCAGCAGCCACTAAAATGTC (L3), TGGAGGATCCTTGGTGGGTTCA (V3-3′a), GGATGGGGATCCTGCTACAAGTC (L4), GGAAGGAATTGTGTGCACAGGT (V4-3′a), GGCGCCCTCTGTGTAGTGGCCTTTGGCCCA (3′β-tubulin), and CAGGCTGGTCAATGTGGCAACCAGATCGGT (5′β-tubulin). V2, V3, V4, and J1 primers have been described previously (49).

Quantitative PCR of DNA. Serial dilutions of DNA samples were prepared. PCR (50) was carried out in a total volume of 100 μl consisting of 25 pmoles each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, and 2.5 U Amplitaq (Cetus Corp.). Samples were heated to 94°C for 3 min followed by amplification for 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C. After the last cycle, a final extension step at 72°C for 10 min was done, 25 μl of each PCR reaction was run on 2% agarose gels in Tris-borate-EDTA buffer. Products were visualized by

ethidium bromide staining. Southern hybridization analysis of the gels with Vγ-specific oligonucleotide probes confirmed the identity of bands corresponding to rearranged Vγ-Jγ genes (data not shown).

Quantitation of the target sequence in the initial nucleic acid sample was accomplished by comparison to the amount of product amplified from titrated quantities of plasmid DNA standards containing the same target sequence. The plasmid DNA standard was prepared by adding 1 pg of plasmid to 1 μg of herring sperm DNA, which was then subjected to serial dilution.

Quantitative PCR of RNA. Total RNA was titrated by serial dilution into a solution containing 1 μg/μl *Escherichia coli* rRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN). cDNA was prepared using 200 U Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), primed with 25 pmoles of the antisense oligonucleotide primer in a total volume of 20 μl containing 1 U/μl RNAsin (Promega Biotech), 1 mM each dNTP, 1.5 mM $MgCl_2$ and 1× PCR buffer. This was incubated for 10 min at 23°C, 30–45 min at 42°C, and the enzyme heat inactivated at 99°C for 5 min. Some reactions were done without reverse transcriptase as a control against the presence of contaminating DNA. PCR was performed using the above cDNA mixture diluted to 100 μl by the addition of 1× PCR buffer, 1.5 mM $MgCl_2$, 25 pmoles sense primer, and 2.5 U Amplitaq. Amplification was carried out as described above.

25 μl of each PCR reaction was run on 2% agarose gels in TBE buffer. Products were visualized by ethidium bromide staining. In some cases the DNA was transferred to nitrocellulose followed by hybridization (47) of the blot with ^{32}P-end-labeled unique oligonucleotides internal to the PCR primers (see Fig. 1). Sequences of the probes are as follows: GAGGCTATTCTGGAAGCTCAG (V2-3′b), TATCCCCTTGGGTGCCCTCAGT (V3-3′b), GCGGGAGTGGGACTTGTCTTGTTT (V3C), TCACCTGCACAGACACCTAG (V4-3′b), ACCTGAGCGAACAGAGTCCATGGTCCC (β-tubulin).

Quantitation of the target sequence in the initial nucleic acid sample was accomplished by comparison to the amount of product amplified from known quantities of synthetic RNA standards containing the same target sequence. The results were adjusted to account for different content of mRNA, as determined by a parallel analysis of tubulin transcripts in the RNA samples.

The synthetic RNA standards were generated by transcription of corresponding linearized plasmid DNA templates. For the reactions in Fig. 4 *A*, these sequences corresponded to the unrearranged Vγ3, Vγ4, or Vγ2 genes. For the reactions in Fig. 6 *A*, the templates corresponded to cloned PCR products of L3-V3-J1 or L4-V3-J1 transcripts (see Results). These cloned PCR products were also used as the templates for the reactions in Fig. 6 *B*, except that we substituted the Vγ3-3′ sequences for the Jγ1 sequences in each plasmid.

RNase Protection Assay. The RNase protection assay was a modification of the procedure of Melton et al. (51) described previously (15).

Results

Rearranged Vγ Genes in Fetal and Double-Negative Thymocyte Populations. Initially, we quantitated Vγ gene rearrangement during development. In previous studies (15) we provided evidence that Vγ3 and Vγ4 rearrangements are more abundant in early fetal thymocytes than in adult thymocytes, whereas Vγ2 rearrangements are more frequent in adult than fetal thymocytes (see Fig. 1 *A* for arrangement of these Vγ genes). Those studies employed unfractionated preparations of thymocytes at most time points, hence the majority of the cells at the later timepoints were $CD4^+CD8^+$ phenotype, most of which are in the α/β-TCR^+ lineage. In the present study, we have focused on the $CD4^-CD8^-$ populations, in which most γ/δ cells and progenitor cells are found. Southern blot analysis of EcoRI-digested $CD4^-CD8^-$ thymocyte DNA revealed a pattern of Vγ rearrangements similar to the pattern we initially reported: Vγ2 rearrangements are evident by E15 (day 15 of gestation) and increase during development to maximal levels in the adult (17-kb band in Fig. 1 *B*). Vγ3 rearrangements are evident by E15 and at E18 (day 18), but decrease to undetectable levels by the adult stage (18-kb band in Fig. 1 *C*). Vγ4 rearrangements are less abundant than Vγ3 rearrangements, but follow a similar developmental pattern (17-kb band in Fig. 1 *C*).

The Southern blot analysis fails to provide sufficient quantitative information to compare the extent of rearrangement of different V genes. Therefore, the $CD4^-CD8^-$ thymocyte DNA samples were also subjected to a quantitative PCR analysis, in which rearranged alleles were amplified from titrated samples of thymocyte DNA using a Jγ1 antisense primer (J1) and a sense strand primer from the leader exon of each Vγ gene (L3, L4, or L2 primer; for locations of primers and probes, see Fig. 1 *A* and Materials and Methods). For the purpose of quantitation, plasmid DNAs containing the corresponding rearranged Vγ-Jγ gene were titrated in parallel PCRs. The products of the PCR reactions were separated on an agarose gel, visualized by ethidium bromide staining, and quantitated by comparison to the reference DNA (Fig. 2). This approach allows the determination of the approximate frequency of rearranged genes in the genomic DNA population. The identity of the various bands was confirmed by Southern hybridization analysis, with the use of Vγ-specific oligonucleotide probes (data not shown).

The PCR analysis revealed that Vγ2 rearrangements are very rare in E14 DNA, and increase ~250-fold during subsequent development to reach a maximum at E18 (Fig. 2, plotted in Fig. 3 *A*). Vγ3 and Vγ4 rearrangements are also relatively infrequent at E14, and their abundance increases ~20-fold by E15. But unlike Vγ2 rearrangements, Vγ3 and Vγ4 rearrangements decrease later in development, by ~30- and 10-fold, respectively.

The quantitative PCR assay allowed us to compare the frequencies of different Vγ gene rearrangements at given time points (data summarized in Fig. 3 *B* as percentages of summed Vγ2, Vγ3, and Vγ4 rearrangements). It is striking that Vγ2 rearrangements are relatively rare at E14, being ~1/10 as frequent as Vγ3 rearrangements, but subsequently increase to dramatically exceed either Vγ3 or Vγ4 rearrangements by the late fetal stage and in the adult. Vγ3 rearrangements, which predominate at E14 and E15, decrease to ~1/50 the level of Vγ2 in the adult. Moreover, the comparisons indicated that Vγ3 rearrangements are more abundant (4–10-fold) than Vγ4 rearrangements in the fetal thymocyte DNA

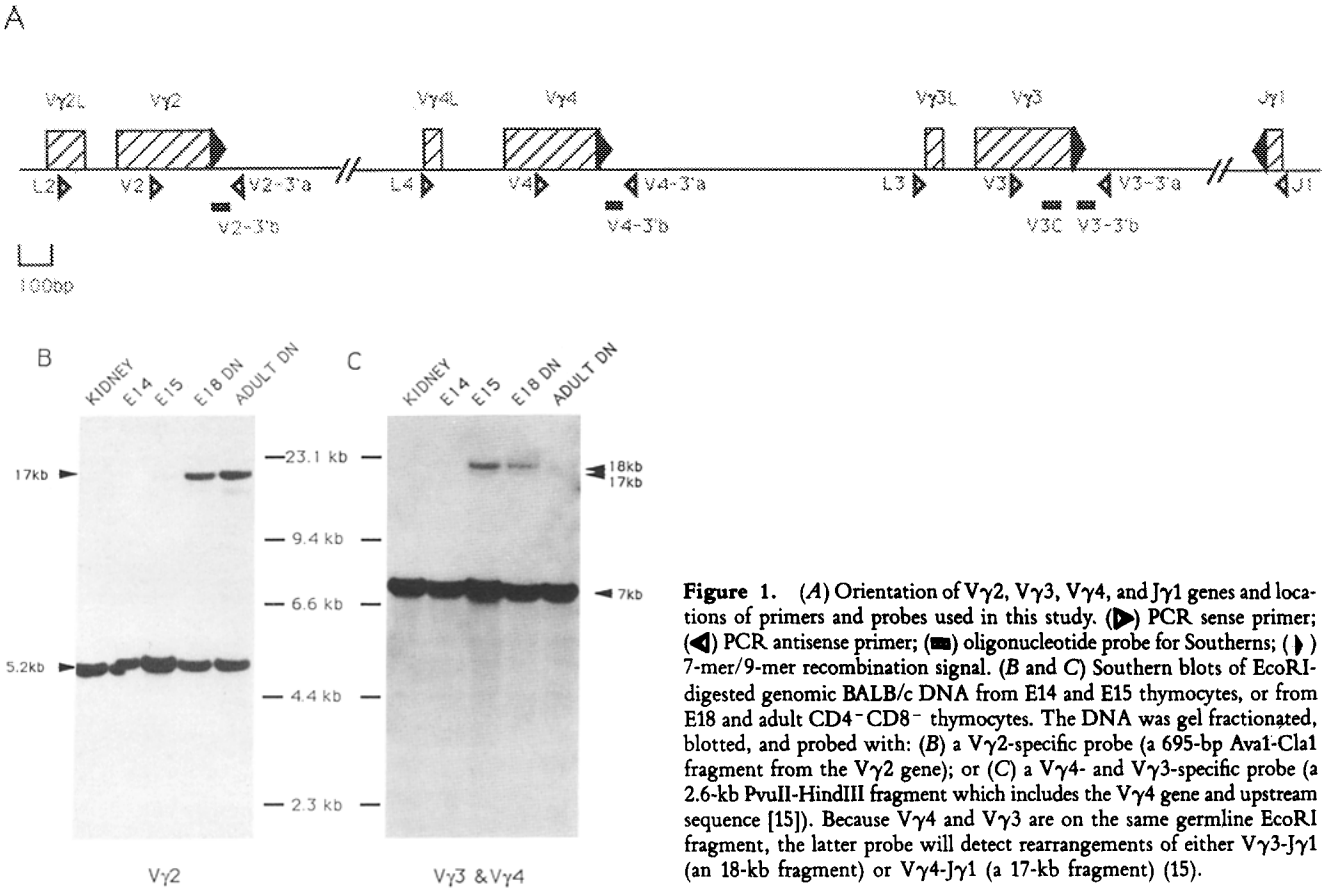

Figure 1. (*A*) Orientation of Vγ2, Vγ3, Vγ4, and Jγ1 genes and locations of primers and probes used in this study. (▶) PCR sense primer; (◀) PCR antisense primer; (▬) oligonucleotide probe for Southerns; (◗) 7-mer/9-mer recombination signal. (*B* and *C*) Southern blots of EcoRI-digested genomic BALB/c DNA from E14 and E15 thymocytes, or from E18 and adult $CD4^-CD8^-$ thymocytes. The DNA was gel fractionated, blotted, and probed with: (*B*) a Vγ2-specific probe (a 695-bp AvaI-ClaI fragment from the Vγ2 gene); or (*C*) a Vγ4- and Vγ3-specific probe (a 2.6-kb PvuII-HindIII fragment which includes the Vγ4 gene and upstream sequence [15]). Because Vγ4 and Vγ3 are on the same germline EcoRI fragment, the latter probe will detect rearrangements of either Vγ3-Jγ1 (an 18-kb fragment) or Vγ4-Jγ1 (a 17-kb fragment) (15).

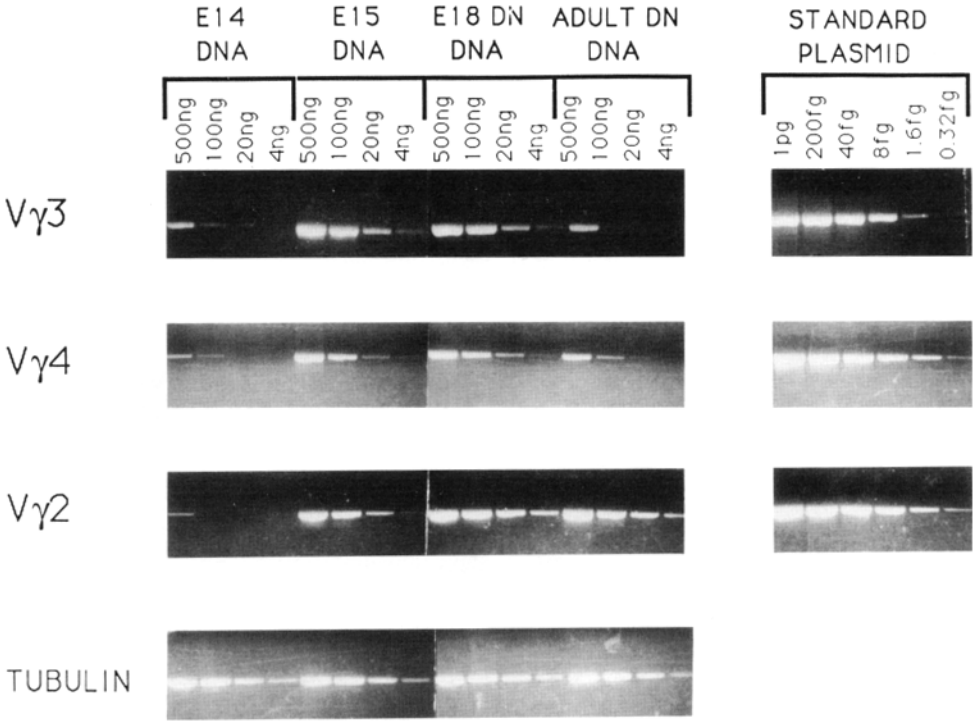

Figure 2. Quantitative PCR analysis of Vγ rearrangements to Jγ1 in thymocyte genomic DNA samples. Dilutions of genomic DNA or plasmid DNA standards were used as templates for PCR. Above each lane is the amount of DNA included in the initial reaction. To amplify rearranged γ genes, the Jγ1 antisense primer and the L3, L4, or L2 sense primers (Fig. 1 *A*) were used. Control PCR using the 5′ and 3′ β-tubulin primers were performed to normalize for the amount of genomic DNA in a sample. 25 μl of each reaction was run on ethidium bromide-stained 2% agarose gels. The size of each product was as predicted: Vγ3, 520 bp; Vγ4, 630 bp; Vγ2, 550 bp; and tubulin, 310 bp. As a control, PCR with each set of primers was also performed using herring sperm DNA instead of template, and yielded no detectable product (not shown).

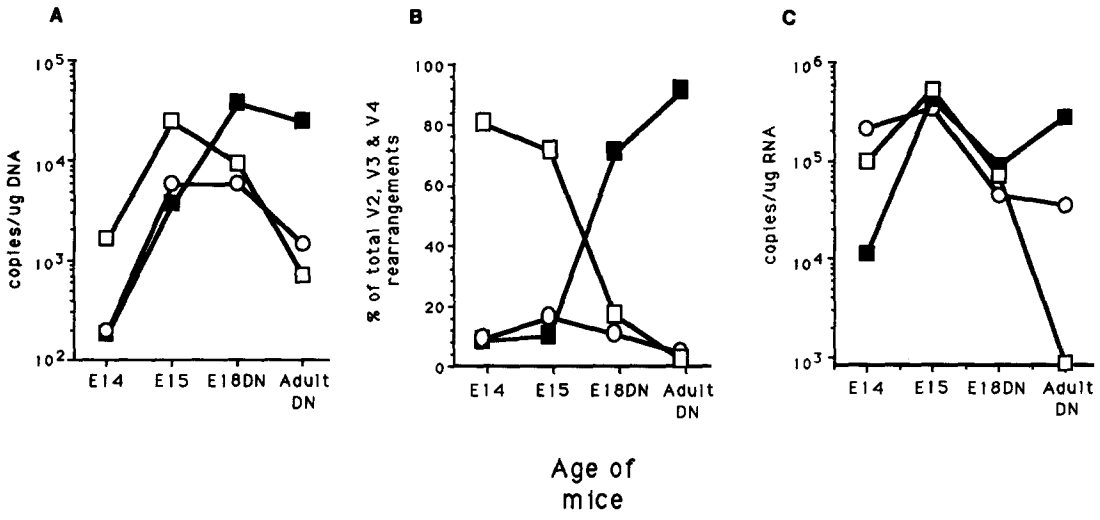

Figure 3. Summary of rearrangements (A and B) and sterile transcripts (*C*) of Vγ2 (■), Vγ3 (□), and Vγ4 (○) genes at different stages of ontogeny. The abundance of Vγ rearrangements is expressed either as (*A*) number of copies/μg DNA, or (*B*) as a percentage of summed Vγ2, Vγ3, and Vγ4 rearrangements. Sterile transcripts are presented as the number of copies/μg total RNA. Quantitative densitometry was used to determine the values from a single RNA and a single DNA experiment, which are depicted in the figure. The results were corroborated in at least one additional complete experiment of each type. The levels of sterile Vγ rearrangements and transcripts at E14 may be slightly underestimated, because of contamination of this sample with thymic capsule cells (see Materials and Methods).

samples. Thus, the relative frequencies of the different Vγ rearrangements correspond reasonably well with the frequencies of Vγ expressing cells at the different time points (see Discussion). The analyses presented here corroborate and considerably extend our earlier results showing that Vγ genes are rearranged differentially during development.

The Abundance of Sterile Vγ Transcripts Correlates with Subsequent Rearrangement Patterns. To determine if transcription is correlated with ordered Vγ gene rearrangement, we developed a PCR-based assay to detect transcripts of unrearranged Vγ genes (subsequently called sterile Vγ transcripts) in fetal and adult thymocytes. Total RNA was isolated from populations of thymocytes prepared identically to those used for the previously described DNA analysis. Using titrated samples of RNA, reverse transcription of cDNA corresponding to sterile Vγ transcripts was primed with antisense oligonucleotides corresponding to sequences 3′ of the unrearranged Vγ2, Vγ3, or Vγ4 genes (Fig. 1 *A*, V2-3′a, V3-3′a, and V4-3′a), which are absent from the corresponding rearranged transcripts. The sterile transcripts were amplified with the same downstream primer and a corresponding upstream sense primer (either in the Vγ coding exon or in the case of Vγ3, the leader exon of the Vγ3 gene). To determine the amounts of the transcripts, synthetic RNAs corresponding to each unrearranged Vγ gene were titrated in parallel reverse transcription/PCRs (see Materials and Methods). PCR products were visualized on Southern blots probed with end-labeled oligonucleotides corresponding to sequences 3′ of the Vγ gene, but internal to the primers used for the PCRs (Fig. 1 *A*, V2-3′b, V3-3′b, and V4-3′b). In addition, the level of the ubiquitously expressed β-tubulin transcript in each RNA sample was determined in parallel quantitative PCRs, to normalize for the amount of mRNA initially added to the PCR reaction. Two controls indicate that contamination of the RNA samples with genomic DNA was not responsible for the observed bands. In the case of Vγ2 and Vγ4, PCRs run in parallel in which reverse transcriptase was omitted in the initial step yielded no bands (Fig. 4 *B*). In the case of the Vγ3 PCR, the primers spanned an intron. No product of the size of the unspliced 550-bp genomic DNA fragment was observed (Fig. 4 *A*). Note that in both Vγ3 and Vγ4 PCR, fragments smaller than the expected fragments were observed in addition to the expected product. This has been reported in other reverse transcription/PCR experiments (52, 53) and may result from aberrant initiation of reverse transcription within the transcript.

As shown in Fig. 4 *A* and summarized in Fig. 3 *C*, sterile Vγ3 transcripts were relatively abundant in E14 thymocytes, increased severalfold at E15, and diminished dramatically (>100-fold) to undetectable levels by the adult stage. The sterile Vγ4 transcript showed a similar pattern, although the decline in levels between the early fetal and adult stage was less precipitous (~10-fold) than that of the sterile Vγ3 transcript. In contrast, sterile Vγ2 transcripts showed the opposite pattern, in that they were very low at E14 and increased ~20–30-fold by E15 to the level they maintained in the adult stage. Considering that increased accessibility of an unrearranged gene is expected to precede rearrangement of the gene, the changes in abundance of sterile transcripts correlate very well with the observed patterns of subsequent Vγ2, Vγ3, and Vγ4 rearrangements during ontogeny (compare Fig. 3, *B* and *C*).

It might be argued that the virtual absence of sterile Vγ3 transcripts in adult double-negative thymocytes is due to the

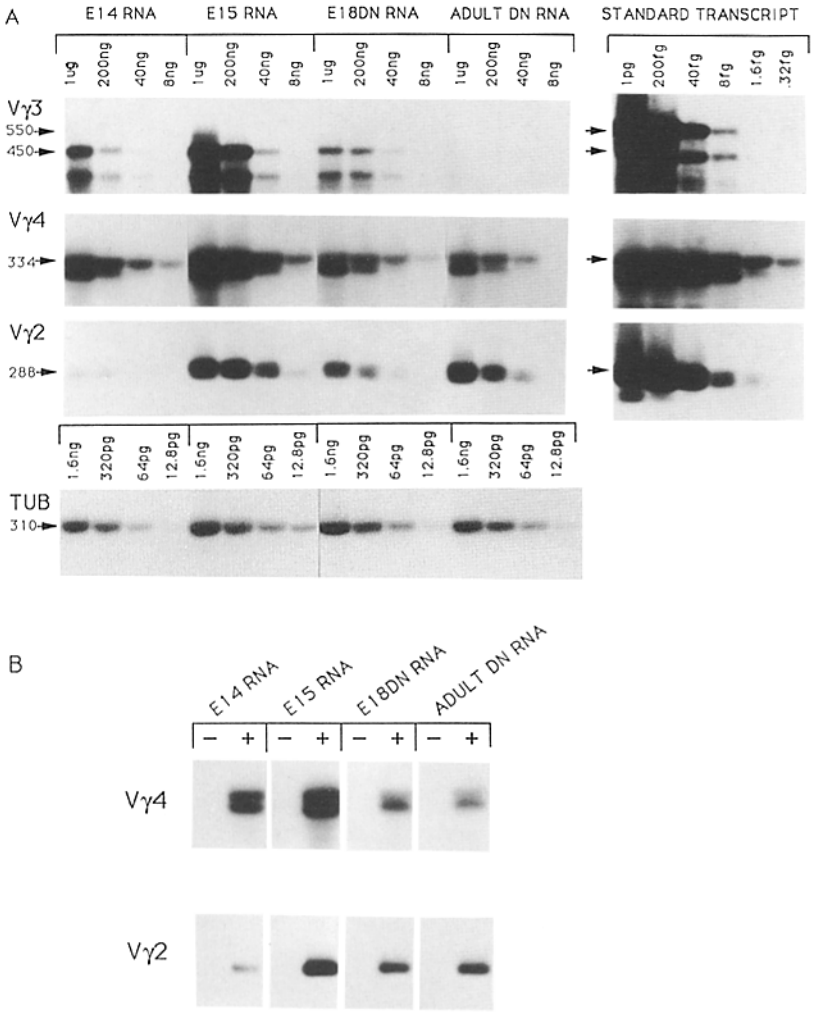

Figure 4. Quantitative PCR to detect sterile Vγ transcripts in total RNA from thymocytes (see text and Materials and Methods). (*A*) Dilutions of total RNA from thymocytes, or of in vitro synthesized standard RNAs, were used as templates, with antisense primers 3′ of each unrearranged Vγ gene (3′a primers, Fig. 1 *A*), to direct cDNA synthesis. The sterile transcripts were amplified with the same downstream primer and an upstream V-specific primer (L3 for Vγ3, V4 for Vγ4, and V2 for Vγ2 [see Fig. 1 *A*]). The products were gel-fractionated, blotted, and hybridized with oligonucleotide probes corresponding to sequences 3′ of the V gene but 5′ of the primer used for PCR (3′b probes in Fig. 1 *A*). The sizes of the products as predicted from the sequences are: Vγ3, 450 bp; Vγ4, 334 bp; Vγ2, 288 bp; and TUB, 310 bp. The size of the larger V3 product (450 bp) indicates it is derived from a correctly spliced transcript. DNA contamination of the RNA sample would have yielded a larger (550 bp) fragment, due to the presence of the intron. The standard plasmid for Vγ3 in this experiment is constructed from genomic DNA, and therefore includes the intron. (*B*) Dependence of the PCRs on reverse transcriptase. For Vγ2 and Vγ4 PCRs, reactions were performed with 1 μg total RNA (same samples as used above) with (+) or without (−) the addition of reverse transcriptase in the initial reaction.

deletion of most of the unrearranged Vγ3 gene segments from the thymocyte population, which would result from rearrangement of Vγ2 (or Vγ4) to Jγ1 in most of the cells. This does not appear to be the case, since a strong band corresponding to the unrearranged Vγ3 and (Vγ4) gene is present in Southern blots of DNA from adult $CD4^-CD8^-$ thymocytes (Fig. 1 *C*). Using the quantitative PCR assay, we found only a twofold or smaller reduction in germline-configuration Vγ3 genes in adult $CD4^-CD8^-$ thymocyte DNA compared to fetal thymocyte DNA (data not shown).

It might also be argued that the sterile Vγ transcripts we detect originate in mature γ/δ^+ cells, from alleles that remain unrearranged, rather than in the progenitors of γ/δ^+ cells. To address this possibility, the PCR analysis was repeated with samples of RNA from E15 and adult double-negative thymocyte populations from which $CD3^+$ cells had been removed by cell sorting. The pattern and amounts of sterile transcripts in these cells was similar to those in the corresponding unsorted thymocyte populations (Table 1). These results indicate that TCR-negative cells produce a substantial fraction of the sterile transcripts we detect.

The Vγ4 Gene Is Transcribed in Cells in which Vγ3 Is Rearranged. The accessibility model, when applied to Vγ gene rearrangement, predicts that not all of the Vγ genes in the Cγ1 cluster will be accessible in a given progenitor cell. The model makes no predictions concerning whether all the Vγ genes in a cell become accessible later, after the cell has differentiated into a mature γ/δ^+ cell, but the issue is pertinent for understanding the possible mechanisms by which rearrangement is regulated. The following studies, based on a chance observation, demonstrate that the Vγ4 gene is accessible in

Table 1. *Sterile Transcript Levels in $CD3^-$ Thymocyte RNA*

Age of mice	Copies/μg	
	Vγ2	Vγ3
E15	5.7×10^5	3.4×10^5
Adult DN*	1.2×10^5	$<3.6 \times 10^3$

* DN, double-negative.

$V\gamma3^+$ cells, but may be inaccessible in progenitors of $V\gamma3^+$ cells.

In experiments to determine the start sites of $V\gamma$ transcripts in a $V\gamma3^+$ cell line, 7.17A2, we found evidence of transcripts containing the $V\gamma4$ leader exon (L4) sequences, but not the $V\gamma4$ coding exon sequences. Since $V\gamma4$ is not rearranged in 7.17A2, we suspected that these transcripts might consist of L4 sequences spliced to the $V\gamma3$ coding exon sequences. cDNA from 7.17A2 RNA was subjected to PCR amplification using either the L3 (V3 leader exon) or L4 sense primer and the J1 antisense primer. Products of both reactions (i.e., using the the L3/J1 or L4/J1 primer pairs) were easily detected and were cloned and sequenced. The $V\gamma3$-$J\gamma1$ junctional sequences of each product corresponded to the functional, canonical s-IEL sequence (4). As expected, in the L3/J1 product, L3 was correctly spliced to $V\gamma3$. The L4/J1 product consisted of L4 correctly spliced to the $V\gamma3$ sequences (54 and data not shown). The origin of the L4-$V\gamma3$-$J\gamma1$ transcript is presumably a large primary transcript that originates upstream of L4 and extends through $V\gamma3$ and the $J\gamma1$-$C\gamma1$ regions (Fig. 5 *A*). A similar case has been reported where $V\beta8.2$ transcripts were shown to frequently use the upstream $V\beta5.1$ leader exon in α/β T cells (55).

The relative abundance of transcripts of the L4-$V\gamma3$-$J\gamma1$ and L3-$V\gamma3$-$J\gamma1$ types in 7.17A2 were quantitated with the use of the RNase protection assay and in vitro synthesized RNA probes corresponding to each cloned PCR product described above. As shown in Fig. 5 *B*, fragments consistent with protection of the probes by both L3-$V\gamma3$ and L4-$V\gamma3$ transcripts were observed with both probes. As determined by densitometric analysis, ~19% of all the $V\gamma3$-$J\gamma1$ transcripts use L4, whereas most of the remaining transcripts use L3. The results indicate that in this cell line an appreciable fraction of $V\gamma3$ transcripts originate from a larger primary transcript that includes L4. Hence, the $V\gamma3$ and $V\gamma4$ genes on the same chromosome must both be accessible to RNA polymerase.

To determine if this is also the case in freshly isolated thymocytes, cDNA from E15 thymocyte RNA was subjected to quantitative PCR, using J1 as a downstream primer, and L3 or L4 as upstream primers. $V\gamma3$-containing PCR products were detected by hybridization with a $V\gamma3$-oligonucleotide probe (V3C, Fig. 1 *A*). The analysis revealed that ~22%

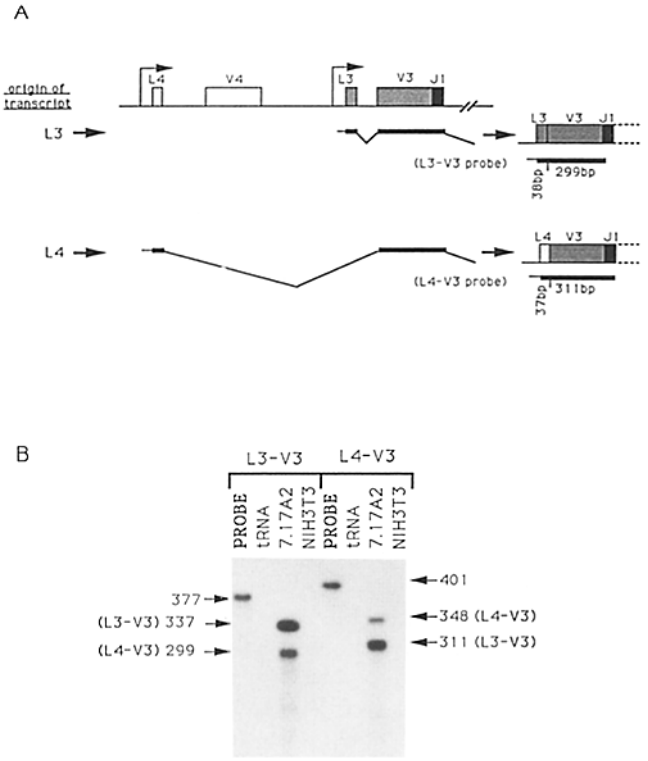

Figure 5. Some $V\gamma3$ transcripts arise by splicing of L4 sequences to $V\gamma3$. (*A*) Schematic representation of RNA splicing to generate $V\gamma3^+$ transcripts employing the L4 or L3 sequences. (*B*) RNase protection assay to detect L3-V3 and L4-V3 transcripts in the 7.17A2 s-IEL line. (*Arrows*) Protected bands, corresponding to the indicated products, and undigested probes. The probes used for RNase protection are shown in (*A*) under the corresponding transcripts. Labeled probes were hybridized with 2 μg of total RNA from 7.17A2 cell lines, from negative control NIH3T3 fibroblastic cells, or with 2 μg yeast tRNA, and digested with RNase. Note that the smaller bands in each case correspond to protection of the V3 exon alone. During cloning of the probes, exonuclease digestion removed different amounts from the 3′ end, leaving the L3-V3 probe with 4 and the L4V3 probe with 16 bases of $J\gamma1$ sequence. This accounts for the difference in size between the lower bands protected with each probe.

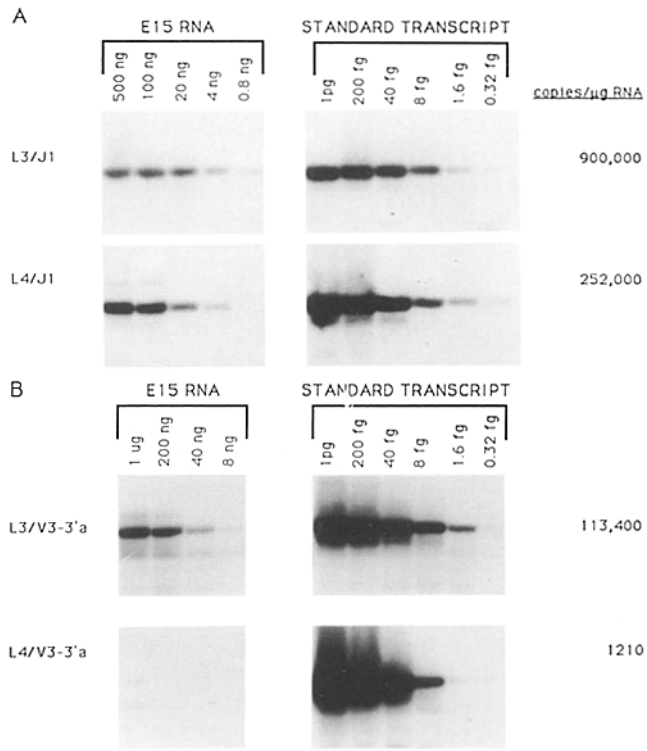

Figure 6. Usage of L4 and L3 in transcripts of unrearranged genes in E15 fetal thymocytes. Transcripts of rearranged (*A*), or unrearranged (*B*) $V\gamma3$ genes were detected by PCR amplification of $J\gamma1$-primed (*A*) or V3–3′a-primed (*B*) cDNA with L4 or L3 upstream primers. Gel-fractionated products were blotted and hybridized with the $V\gamma3$ oligonucleotide probe. Comparison of titrated E15 RNA with titrated in vitro synthesized standard transcripts (see Materials and Methods) allowed the determination of the number of copies/μg total RNA, indicated to the right of the figure.

of rearranged Vγ3 transcripts in E15 cells are of the L4-Vγ3-Jγ1 type (Fig. 6 *A*). Therefore, as in the 7.17A2 cell line, a substantial fraction of rearranged Vγ3 transcripts from E15 thymocytes appear to result from the processing of a transcript that initiates upstream of the Vγ4 gene, indicating that both the Vγ3 and Vγ4 genes must be accessible to RNA polymerase on a significant fraction of the rearranged chromosomes.

The Vγ4 Gene May Be Inaccessible in Progenitors of γ/δ Cells. The preceding results raise the question whether the Vγ4 and Vγ3 genes are both accessible on the same chromosome before rearrangement, which would argue that rearrangement of Vγ3 vs. Vγ4 is not controlled by differential accessibility. If both genes are accessible on the same chromosome, it might be expected that the sterile Vγ3 transcripts in fetal thymocytes, like the rearranged transcripts, would commonly initiate upstream of the Vγ4 gene and include the L4 exon spliced to the Vγ3 exon. To examine this possibility, we employed a PCR assay similar to that used in the previous experiment, except that in order to amplify transcripts of the unrearranged genes, we used the V3–3'a downstream primer instead of the J1 primer. As before, the upstream primers were either the L3 or the L4 primers. Using this method, we could determine the abundance of sterile transcripts containing L4 spliced to Vγ3. In contrast to what we observed in the case of rearranged transcripts, L4-Vγ3 sterile transcripts were much rarer (~1/100th the level) than L3-Vγ3 sterile transcripts in E15 thymocytes (Fig. 6 *B*). These data indicate that very few of the sterile Vγ3 transcripts arise by splicing of L4 to Vγ3. A possible explanation is that the long primary transcripts that initiate upstream of Vγ4 and include Vγ3 are not synthesized in progenitors of Vγ3 cells, perhaps because the Vγ4 gene is inaccessible in these cells. According to this possibility, the Vγ4 gene is inaccessible in the progenitors of $V\gamma3^+$ cells, but becomes accessible in $V\gamma3^+$ cells after rearrangement, allowing the production of L4-V3-J1 transcripts. Of course, the Vγ4 gene must also be accessible in the progenitors of $V\gamma4^+$ cells.

Discussion

Expression of Germline Vγ Genes Correlates with Rearrangement. The central new conclusion of the present study is that the developmental pattern of Vγ gene rearrangements is strikingly correlated with the corresponding pattern of sterile Vγ transcripts. This is the first reported instance where the pattern of V gene rearrangement during development has been correlated with transcription of the unrearranged V genes. Another novel aspect of our results is the demonstration of instances where the cessation of rearrangement is correlated with the disappearance of the corresponding sterile transcript, as in the case of Vγ3 and Vγ4 rearrangements. Previous examples have documented correlations between the onset of rearrangements and the appearance of sterile transcripts (see introductory section), as we also report in the case of Vγ2 rearrangements. The fact that different Vγ gene rearrangements follow opposite trends during development, which are positively correlated with the abundance of the corresponding sterile Vγ transcripts, argues strongly against a chance relationship. Differences in the steady state level of a given transcript at different stages of development are likely to reflect differences in transcription rates, although we cannot rule out the unlikely possibility that degradation rates change specifically during development.

The correlation between transcription and rearrangement argues in favor of a model in which differential accessibility regulates Vγ gene rearrangement. The results of our studies of rearranged versus sterile Vγ3 transcripts are also consistent with the accessibility model. Initially, we found that Vγ3-Jγ1 transcripts in an s-IEL cell line and in fetal thymocytes often employ the upstream Vγ4 leader exon, indicating that both the Vγ3 and Vγ4 genes must be accessible in many of the cells with rearranged Vγ3 genes. In contrast, sterile Vγ3 transcripts in fetal thymocytes employ the Vγ4 leader exon only rarely. As an explanation for these results, we propose that essentially all Vγ3 sterile transcripts derive from primary transcripts initiating at the Vγ3 promoter, rather than from larger transcripts that include L4. The larger sterile transcripts would not be produced if the L4/Vγ4 gene is inaccessible in the progenitors of Vγ3 cells, and/or if the Vγ4 promoter is inactive in these cells. An alternative possibility, that we have not ruled out, is that the large sterile transcript is produced, but L4 fails to be spliced to Vγ3. We think this is unlikely, however, since other splicing events occur normally in sterile transcripts, for example L3 to Vγ3 (Figs. 4 and 6) and L4 to Vγ4 (data not shown).

An Accessibility Model of Vγ Gene Rearrangement. An attractive version of the accessibility model as applied to Vγ gene rearrangement is that Vγ gene accessibility is controlled by selective activation of the Vγ gene promoters. Gene accessibility, in turn, controls differential gene rearrangement. The fact that the promoter regions of Vγ2, Vγ3, and Vγ4 show no obvious sequence relatedness (Doherty and Raulet, unpublished observations), is consistent with the possibility that they are independently regulated. After gene rearrangement and/or maturation of the γ/δ cell, transcription of other Vγ genes in the cell apparently occurs, as shown by the splicing of the Vγ4 leader exon to Vγ3 in rearranged transcripts. Among other possibilities, this may occur because rearrangement brings the downstream Cγ1 enhancer (56, 57) nearer to the previously silent promoters, thus activating them. Another possibility is that each V gene has two types of promoter elements, one of which is selectively activated to produce the sterile transcripts, and a second promoter element, active in all mature γ/δ cells, used to produce the mature transcripts.

It should be emphasized that the promoter activation model does not require that accessibility be controlled by transcription per se. The binding of transcription factors to a promoter (or enhancer) may directly render a gene accessible to recombinase. This may occur in the absence of transcription in some cases (40, 41), although in the present case transcription does accompany recombination. Hence, transcription is often an indication of gene accessibility, but does not necessarily cause accessibility.

Control of Vγ gene accessibility by promoter sequences

is an attractive model, but there is no direct evidence to date implicating the promoter sequences in the process. It is also possible that accessibility of these genes is controlled by *cis*-acting DNA elements distinct from the promoters.

Mechanisms Activating Gene Rearrangement in the Thymus. It is interesting to speculate on how programming of gene rearrangement may be accomplished mechanistically within the thymus. Evidence indicates that the production of $V\gamma3^+$ cells depends on the fetal origin of both the hematopoietic stem cells and the thymic stroma (24; J. Allison, personal communication). The requirement for fetal stem cells may indicate that only these cells are susceptible to thymic stromal signals that activate the accessibility of the Vγ3 gene. The nature of these putative thymic stromal signals is not known, but may correspond to stromal cell surface ligands or cytokines that are elaborated in a stage-specific fashion. An interesting possibility is that the signals that activate Vγ gene rearrangement also selectively activate the expression of other genes that may function specifically in a given γ/δ cell subtype, such as those encoding tissue-specific homing receptors.

Ordered Rearrangement of Vγ Genes in Ontogeny. Although the ordered developmental pattern of Vγ gene rearrangements was described previously (15), the present study provides quantitative comparisons of the extent of each Vγ gene rearrangement in the population. This new information is important because it allows us to ascertain whether there are discrepancies between the extent of rearrangement of each Vγ gene and the representation of cells expressing a Vγ product on the cell surface. Such discrepancies might be used to argue for selection events acting on nascent γ/δ cells.

In fact, the extent of Vγ gene rearrangements in the early fetal and adult stages, when adjusted for the rates at which each type of rearrangement is found to be productive, roughly corresponds to the relative frequencies of cells expressing a given Vγ product on the cell surface. The frequencies of Vγ3 and Vγ4 rearrangements in the fetal thymus that are productive (~60%) is much higher than the frequency of Vγ2 rearrangements that are productive (~10%) (58). Using these approximate values, it can be calculated that the ratios of productive rearrangements at E14 is ~50Vγ3:6Vγ4:1Vγ2, which fits with the reported predominance of $V\gamma3^+$ cells in the early fetal thymus (20, 21). At E18, adjustment of the values for the rates of productive rearrangements yields a ratio of ~2Vγ3:1Vγ4:1Vγ2, consistent with the pattern of Vγ surface expression found in one study (21). In the adult $CD4^-CD8^-$ population, the ratio, calculated from our data using the reported approximate frequencies of adult thymic Vγ rearrangements that are productive (50% of Vγ3, 37% of Vγ4, and 21% of Vγ2 [58]), is ~1Vγ3:2Vγ4:15Vγ2. These values fit well with the findings that $V\gamma2^+$ cells greatly outnumber $V\gamma3^+$ and $V\gamma4^+$ cells in the adult (20, 21).

Hence, there is a good concordance between the extent of V gene rearrangement and the abundance of $V\gamma^+$ cells. In contrast, previous studies demonstrated that the levels of rearranged Vγ/Jγ1 transcripts correlate poorly with the representation of cells expressing a given Vγ product on the cell surface (15, 59). But the levels of transcripts of the rearranged genes can be influenced by numerous variables and are not necessarily correlated with the extent of DNA rearrangements. For example, Vγ2 genes are frequently rearranged in α/β T cells, but are usually transcriptionally silent (15, 16).

Programmed Gene Rearrangement Versus Selection. A key implication of our results is that it is not necessary to invoke selection events that may act on nascent γ/δ cells (60–62) to explain the ordered usage of Vγ genes. Rather, the ordered production of γ/δ cells may be accomplished by regulating gene rearrangements, and by coordinate selective activation of other genes that may function specifically in γ/δ cells of a given subtype. Nevertheless, whereas our results represent an argument for regulation of gene rearrangement, it should be pointed out that selection and regulated gene rearrangements are not mutually exclusive.

We have focused our attention on regulation of the simplest TCR gene family, the TCR-γ genes, and only on a subset of the known Vγ and Cγ genes. The overall regulation of the γ gene utilization is certainly more complex than we have referred to here, but by focusing on a relatively simple subset of the genes, which follow a striking developmental rearrangement pattern, we hope to learn more about the mechanisms that control rearrangement of TCR and Ig genes and the production of specialized subtypes of lymphocytes. Future studies will address the thymic signals which stimulate rearrangement of one or another Vγ gene, as well as the intracellular mechanisms that regulate gene accessibility and subsequent rearrangement.

The authors would like to thank Drs. Ellen Robey, James Allison, Astar Winoto, and David Asarnow for helpful discussions and critical reading of the manuscript; Robert West and Christopher Grimsrud for excellent technical assistance; and Dr. Isabel Correa for advice on PCR.

This work was supported by National Institutes of Health grant RO1 AI-31650. J. Goldman is a visiting scholar from the Massachusetts Institute of Technology.

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Received for publication 23 September 1992 and in revised form 2 December 1992.

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